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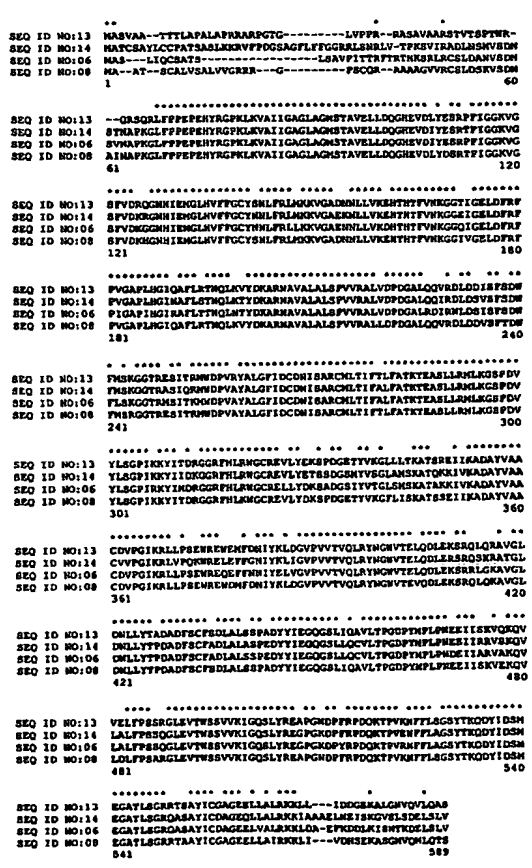
(54) Title: CAROTENOID BIOSYNTHESIS ENZYMES

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a carotenoid biosynthetic enzyme. The invention also relates to the construction of a chimeric gene encoding all or a portion of the carotenoid biosynthetic enzyme, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the carotenoid biosynthetic enzyme in a transformed host cell.



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TITLE

CAROTENOID BIOSYNTHESIS ENZYMES

This application claims the benefit of U.S. Provisional Application No. 60/083.042, filed April 24, 1998.

FIELD OF THE INVENTION

This invention is in the field of plant molecular biology. More specifically, this invention pertains to nucleic acid fragments encoding enzymes of the carotenoid biosynthesis pathway in plants and seeds.

BACKGROUND OF THE INVENTION

Plant carothenoids are orange and red lipid-soluble pigments found embedded in the membranes of chloroplasts and chromoplasts. In leaves and immature fruits the color is masked by chlorophyll but in later stages of development these pigments contribute to the bright color of flowers and fruits. Carothenoids protect against photoxidation processes and harvest light for photosynthesis. The carotenoid biosynthesis pathway leads to the production of abscisic acid with intermediaries useful in the agricultural and food industries as well as products thought to be involved in cancer prevention. (Bartley, G. E., and Scolnik, P. A. (1995) *Plant Cell 7*:1027-1038).

Phytoene desaturase transforms phytoene to zeta carotene via phytofluene. cDNAs encoding this bifunctional enzyme have been identified from bacteria, peppers, corn, Arabidopsis and Narcissus. The lightly colored zeta-carotene is converted to neurosporene by the zeta-carotene desaturase enzyme (carotene 7, 8 desaturase; EC 1.134.99.30). cDNAs encoding zeta carotene desaturase have been identified in bacteria, corn, Narcissus, tomato, Arabidopsis and pepper. Neurosporene is further desaturated into lycopene. Lycopene may have one of two different fates: through the action of lycopene epsilon cyclase it may become alpha carotene, or it may be transformed into beta carotene by lycopene cyclase. Beta-carotene dehydroxylase converts beta-carotene into zeaxanthin. Zeaxanthin epoxidase transforms zeaxanthin into violxanthin and eventually absisic acid.

Zeaxanthin is the bright orange product highly prized as a pigmenting agent for animal feed which makes the meat fat, skin, and egg yolks a dark yellow (Scott, M. L. et al. (1968) *Poultry Sci. 47*:863-872). Gram per gram, zeaxanthin is one of the best pigmenting compounds because it is highly absorbable. Yellow corn, which produces one of the best ratios of lutein to zeaxanthin contains in average 20 to 25 mg of xanthophyll per kg while marigold petals yield 6,000 to 10,000 mg/kg.

SUMMARY OF THE INVENTION

The instant invention relates to isolated nucleic acid fragments encoding carotenoid biosynthetic enzymes. Specifically, this invention concerns an isolated nucleic acid fragment encoding a zeta carotene desaturase or a phytoene desaturase. In addition, this invention relates to a nucleic acid fragment that is complementary to the nucleic acid fragment encoding zeta carotene desaturase or phytoene desaturase.

An additional embodiment of the instant invention pertains to a polypeptide encoding all or a substantial portion of a carotenoid biosynthetic enzyme selected from the group consisting of zeta carotene desaturase and phytoene desaturase.

In another embodiment, the instant invention relates to a chimeric gene encoding a zeta carotene desaturase or a phytoene desaturase, or to a chimeric gene that comprises a nucleic acid fragment that is complementary to a nucleic acid fragment encoding a zeta carotene desaturase or a phytoene desaturase, operably linked to suitable regulatory sequences, wherein expression of the chimeric gene results in production of levels of the encoded protein in a transformed host cell that is altered (i.e., increased or decreased) from the level produced in an untransformed host cell.

In a further embodiment, the instant invention concerns a transformed host cell comprising in its genome a chimeric gene encoding a zeta carotene desaturase or a phytoene desaturase, operably linked to suitable regulatory sequences. Expression of the chimeric gene results in production of altered levels of the encoded protein in the transformed host cell. The transformed host cell can be of eukaryotic or prokaryotic origin, and include cells derived from higher plants and microorganisms. The invention also includes transformed plants that arise from transformed host cells of higher plants, and seeds derived from such transformed plants.

An additional embodiment of the instant invention concerns a method of altering the level of expression of a zeta carotene desaturase or a phytoene desaturase in a transformed host cell comprising: a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a zeta carotene desaturase or a phytoene desaturase; and b) growing the transformed host cell under conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of altered levels of zeta carotene desaturase or phytoene desaturase in the transformed host cell.

An addition embodiment of the instant invention concerns a method for obtaining a nucleic acid fragment encoding all or a substantial portion of an amino acid sequence encoding a zeta carotene desaturase or a phytoene desaturase.

A further embodiment of the instant invention is a method for evaluating at least one compound for its ability to inhibit the activity of a zeta carotene desaturase or a phytoene desaturase, the method comprising the steps of: (a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a zeta carotene desaturase or a phytoene desaturase, operably linked to suitable regulatory sequences; (b) growing the transformed host cell under conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of zeta carotene desaturase or phytoene desaturase in the transformed host cell; (c) optionally purifying the zeta carotene desaturase or the phytoene desaturase expressed by the transformed host cell; (d) treating the zeta carotene desaturase or the phytoene desaturase with a compound to be tested; and (e) comparing the activity of the zeta carotene desaturase or the phytoene desaturase that has

been treated with a test compound to the activity of an untreated zeta carotene desaturase or phytoene desaturase, thereby selecting compounds with potential for inhibitory activity.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description and the accompanying drawings and Sequence Listing which form a part of this application.

Figure 1 depicts the amino acid sequence alignment between the zeta carotene desaturase from soybean clone sr1.pk0022.a12 (SEQ ID NO:6), wheat clone wlm24.pk0031.e9 (SEQ ID NO:8), *Zea mays* (NCBI gi Accession No. 4105563, SEQ ID NO:13) and *Capsicum annuum* (NCBI gi Accession No. 2129927, SEQ ID NO:14). Amino acids which are conserved among all sequences are indicated with an asterisk (*). Dashes are used by the program to maximize alignment of the sequences.

Figure 2 depicts the amino acid sequence alignment between the phytoene desaturase from rice clone rlr6.pk0027.d5 (SEQ ID NO:10), wheat clone wlm12.pk0003.g5 (SEQ ID NO:12) and *Zea mays* (NCBI gi Accession No. 1345838, SEQ ID NO:15). Amino acids which are conserved among all sequences are indicated with an asterisk (*). Dashes are used by the program to maximize alignment of the sequences.

The following sequence descriptions and sequence listings attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

SEQ ID NO:1 is the nucleotide sequence comprising portion of the cDNA insert in clone rls6.pk0080.a9 encoding the N-terminal fifth of a rice zeta-carotene desaturase.

SEQ ID NO:2 is the deduced amino acid sequence of the N-terminal fifth of a rice zeta-carotene desaturase derived from the nucleotide sequence of SEQ ID NO:1.

SEQ ID NO:3 is the nucleotide sequence comprising a portion of the cDNA insert in clone rls6.pk0080.a9 encoding the C-terminal quarter of a rice zeta-carotene desaturase.

SEQ ID NO:4 is the deduced amino acid sequence of the C-terminal quarter of a rice zeta-carotene desaturase. derived from the nucleotide sequence of SEQ ID NO:3.

SEQ ID NO:5 is the nucleotide sequence comprising a portion of the cDNA insert in clone sr1.pk0022.a12 encoding a portion of a soybean zeta-carotene desaturase.

SEQ ID NO:6 is the deduced amino acid sequence of a portion of a soybean zeta-carotene desaturase derived from the nucleotide sequence of SEQ ID NO:5.

SEQ ID NO:7 is the nucleotide sequence comprising the entire cDNA insert in clone wlm24.pk0031.e9 encoding an entire wheat zeta-carotene desaturase.

SEQ ID NO:8 is the deduced amino acid sequence of an entire wheat zeta-carotene desaturase derived from the nucleotide sequence of SEQ ID NO:7.

SEQ ID NO:9 is the nucleotide sequence comprising the entire cDNA insert in clone rlr6.pk0027.d5 encoding an entire rice phytoene desaturase.

SEQ ID NO:10 is the deduced amino acid sequence of an entire rice phytoene desaturase derived from the nucleotide sequence of SEQ ID NO:9.

SEQ ID NO:11 is the nucleotide sequence comprising the entire cDNA insert in clone wlm12.pk0003.g5 encoding a nearly entire wheat phytoene desaturase.

SEQ ID NO:12 is the deduced amino acid sequence of a nearly entire wheat phytoene desaturase derived from the nucleotide sequence of SEQ ID NO:11.

SEQ ID NO:13 is the amino acid sequence of a *Zea mays* zeta carotene desaturase, NCBI gi Accession No. 4105563.

SEQ ID NO:14 is the amino acid sequence of a *Capsicum annuum* zeta carotene desaturase, NCBI gi Accession No. 2129927.

SEQ ID NO:15 is the amino acid sequence of a *Zea mays* phytoene desaturase, NCBI gi Accession No. 1345838.

The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Research 13*:3021-3030 (1985) and in the *Biochemical Journal 219 (No. 2)*:345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized. As used herein, an "isolated nucleic acid fragment" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially affect the functional properties of the resulting transcript vis-à-vis the ability to mediate alteration of gene expression by antisense or co-suppression technology or alteration of the functional properties of the resulting protein molecule. It is therefore understood that the invention encompasses more than the specific exemplary sequences.

For example, it is well known in the art that antisense suppression and co-suppression of gene expression may be accomplished using nucleic acid fragments representing less than the entire coding region of a gene, and by nucleic acid fragments that do not share 100%

sequence identity with the gene to be suppressed. Moreover, alterations in a gene which result in the production of a chemically equivalent amino acid at a given site, but do not effect the functional properties of the encoded protein, are well known in the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, the skilled artisan recognizes that substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C), with the sequences exemplified herein. Preferred substantially similar amino acid fragments of the instant invention are those amino acid fragments whose protein sequences are 95% identical to the protein fragments reported herein. Sequence alignments and percent similarity calculations were performed using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the amino acid sequences was performed using the Clustal method of alignment (Higgins, D. G. and Sharp, P. M. (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

A "substantial portion" of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to afford putative identification of that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol. 215*:403-410; see also www.ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence

comprises enough of the sequence to afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches partial or complete amino acid and nucleotide sequences encoding one or more particular plant proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment that encodes all or a substantial portion of the amino acid sequence encoding the zeta carotene desaturase or the phytoene desaturase proteins as set forth in SEQ ID NOs:2, 4, 6, 8, 10 and 12. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments which are then enzymatically assembled to construct the entire gene. "Chemically synthesized", as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an

organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg, (1989) *Biochemistry of Plants 15*:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The "translation leader sequence" refers to a DNA sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D. (1995) *Molecular Biotechnology 3*:225).

The "3' non-coding sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., (1989) *Plant Cell 1*:671-680.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. Patent No. 5,107,065, incorporated herein by reference). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to sense RNA, antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020, incorporated herein by reference).

"Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

"Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be but are not limited to intracellular localization signals.

A "chloroplast transit peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the chloroplast or other plastid types

present in the cell in which the protein is made. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast transit peptide. A "signal peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the secretory system (Chrispeels, J. J., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* *42*:21-53). If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*) may be added. If the protein is to be directed to the nucleus, any signal peptide present should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys.100*:1627-1632).

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of methods of plant transformation include Agrobacterium-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol. 143*:277) and particle-accelerated or "gene gun" transformation technology (Klein T. M. et al. (1987) *Nature (London) 327*:70-73; U.S. Patent No. 4,945,050, incorporated herein by reference).

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter "Maniatis").

Nucleic acid fragments encoding at least a portion of several carotenoid biosynthetic enzymes have been isolated and identified by comparison of random plant cDNA sequences to public databases containing nucleotide and protein sequences using the BLAST algorithms well known to those skilled in the art. Table 1 lists the proteins that are described herein, and the designation of the cDNA clones that comprise the nucleic acid fragments encoding these proteins.

TABLE 1

Carotenoid Biosynthetic Enzymes

Enzyme	Clone	Plant
Zeta Carotene Desaturase	rls6.pk0080.a9	Rice
	sr1.pk0022.a12	Soybean
	wlm24.pk0031.e9	Wheat
Phytoene Desaturase	rlr6.pk0027.d5	Rice
	wlm12.pk0003.g5	Wheat

The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous proteins from the same or other plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples

of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

For example, genes encoding other zeta carotene desaturases or phytoene desaturases, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired plant employing methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer DNA labeling, nick translation, or end-labeling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part or all of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, two short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. The polymerase chain reaction may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al., (1988) *Proc. Natl. Acad. Sci. USA 85*:8998) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., (1989) *Proc. Natl. Acad. Sci. USA 86*:5673; Loh et al., (1989) *Science 243*:217). Products generated by the 3' and 5' RACE procedures can be combined to generate full-length cDNAs (Frohman, M. A. and Martin, G. R., (1989) *Techniques 1*:165).

Availability of the instant nucleotide and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides can be used to immunize animals to produce polyclonal or monoclonal antibodies with specificity for peptides or proteins comprising the amino acid sequences. These antibodies can be then

be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest (Lerner, R. A. (1984) *Adv. Immunol. 36*:1; Maniatis).

The nucleic acid fragments of the instant invention may be used to create transgenic plants in which the disclosed zeta carotene desaturase or phytoene desaturase are present at higher or lower levels than normal or in cell types or developmental stages in which they are not normally found. This would have the effect of altering the level of lycopene and phytoene in those cells. Manipulation of the zeta carotene desaturase or phytoene desaturase levels in transgenic plants may allow a greater accumulation of lycopene, zeaxanthin and eventually absisic acid.

Overexpression of the zeta carotene desaturase or the phytoene desaturase proteins of the instant invention may be accomplished by first constructing a chimeric gene in which the coding region is operably linked to a promoter capable of directing expression of a gene in the desired tissues at the desired stage of development. For reasons of convenience, the chimeric gene may comprise promoter sequences and translation leader sequences derived from the same genes. 3' Non-coding sequences encoding transcription termination signals may also be provided. The instant chimeric gene may also comprise one or more introns in order to facilitate gene expression.

Plasmid vectors comprising the instant chimeric gene can then constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., (1985) *EMBO J. 4*:2411-2418; De Almeida et al., (1989) *Mol. Gen. Genetics 218*:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

For some applications it may be useful to direct the instant carotenoid biosynthetic enzymes to different cellular compartments, or to facilitate its secretion from the cell. It is thus envisioned that the chimeric gene described above may be further supplemented by altering the coding sequence to encode zeta carotene desaturase or phytoene desaturase with appropriate intracellular targeting sequences such as transit sequences (Keegstra, K. (1989) *Cell 56*:247-253), signal sequences or sequences encoding endoplasmic reticulum localization (Chrispeels, J. J., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol. 42*:21-53), or nuclear localization signals (Raikhel, N. (1992) *Plant Phys. 100*:1627-1632) added and/or with targeting sequences that are already present removed. While the references cited give examples of each of these, the list is not exhaustive and more targeting signals of utility may be discovered in the future.

It may also be desirable to reduce or eliminate expression of genes encoding zeta carotene desaturase or phytoene desaturase in plants for some applications. In order to accomplish this, a chimeric gene designed for co-suppression of the instant carotenoid biosynthetic enzyme can be constructed by linking a gene or gene fragment encoding a zeta carotene desaturase or a phytoene desaturase to plant promoter sequences. Alternatively, a chimeric gene designed to express antisense RNA for all or part of the instant nucleic acid fragment can be constructed by linking the gene or gene fragment in reverse orientation to plant promoter sequences. Either the co-suppression or antisense chimeric genes could be introduced into plants via transformation wherein expression of the corresponding endogenous genes are reduced or eliminated.

The instant zeta carotene desaturase or phytoene desaturase (or portions thereof) may be produced in heterologous host cells, particularly in the cells of microbial hosts, and can be used to prepare antibodies to the these proteins by methods well known to those skilled in the art. The antibodies are useful for detecting zeta carotene desaturase or phytoene desaturase *in situ* in cells or *in vitro* in cell extracts. Preferred heterologous host cells for production of the instant zeta carotene desaturase or phytoene desaturase are microbial hosts. Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct a chimeric gene for production of the instant zeta carotene desaturase or phytoene desaturase. This chimeric gene could then be introduced into appropriate microorganisms via transformation to provide high level expression of the encoded carotenoid biosynthetic enzyme. An example of a vector for high level expression of the instant zeta carotene desaturase or phytoene desaturase in a bacterial host is provided (Example 7).

Additionally, the instant zeta carotene desaturase or phytoene desaturase can be used as targets to facilitate design and/or identification of inhibitors of those enzymes that may be useful as herbicides. This is desirable because the zeta carotene desaturase and the phytoene desaturase described herein catalyze various steps in carotenoid biosynthesis. Accordingly, inhibition of the activity of either one or both of the enzymes described herein could lead to inhibition plant growth. Thus, the instant zeta carotene desaturase or phytoene desaturase could be appropriate for new herbicide discovery and design.

All or a substantial portion of the nucleic acid fragments of the instant invention may also be used as probes for genetically and physically mapping the genes that they are a part of, and as markers for traits linked to those genes. Such information may be useful in plant breeding in order to develop lines with desired phenotypes. For example, the instant nucleic acid fragments may be used as restriction fragment length polymorphism (RFLP) markers. Southern blots (Maniatis) of restriction-digested plant genomic DNA may be probed with the nucleic acid fragments of the instant invention. The resulting banding patterns may then be subjected to genetic analyses using computer programs such as MapMaker (Lander et al.,

(1987) *Genomics 1*:174-181) in order to construct a genetic map. In addition, the nucleic acid fragments of the instant invention may be used to probe Southern blots containing restriction endonuclease-treated genomic DNAs of a set of individuals representing parent and progeny of a defined genetic cross. Segregation of the DNA polymorphisms is noted and used to calculate the position of the instant nucleic acid sequence in the genetic map previously obtained using this population (Botstein, D. et al., (1980) *Am. J. Hum. Genet. 32*:314-331).

The production and use of plant gene-derived probes for use in genetic mapping is described in R. Bernatzky, R. and Tanksley, S. D. (1986) *Plant Mol. Biol. Reporter 4(1)*:37-41. Numerous publications describe genetic mapping of specific cDNA clones using the methodology outlined above or variations thereof. For example, F2 intercross populations, backcross populations, randomly mated populations, near isogenic lines, and other sets of individuals may be used for mapping. Such methodologies are well known to those skilled in the art.

Nucleic acid probes derived from the instant nucleic acid sequences may also be used for physical mapping (i.e., placement of sequences on physical maps; *see* Hoheisel, J. D., et al., In: *Nonmammalian Genomic Analysis: A Practical Guide*, Academic press 1996, pp. 319-346, and references cited therein).

In another embodiment, nucleic acid probes derived from the instant nucleic acid sequences may be used in direct fluorescence *in situ* hybridization (FISH) mapping (Trask, B. J. (1991) *Trends Genet. 7*:149-154). Although current methods of FISH mapping favor use of large clones (several to several hundred KB; see Laan, M. et al. (1995) *Genome Research 5*:13-20), improvements in sensitivity may allow performance of FISH mapping using shorter probes.

A variety of nucleic acid amplification-based methods of genetic and physical mapping may be carried out using the instant nucleic acid sequences. Examples include allele-specific amplification (Kazazian, H. H. (1989) *J. Lab. Clin. Med. 114(2)*:95-96), polymorphism of PCR-amplified fragments (CAPS; Sheffield, V. C. et al. (1993) *Genomics 16*:325-332), allele-specific ligation (Landegren, U. et al. (1988) *Science 241*:1077-1080), nucleotide extension reactions (Sokolov, B. P. (1990) *Nucleic Acid Res. 18*:3671), Radiation Hybrid Mapping (Walter, M. A. et al. (1997) *Nature Genetics 7*:22-28) and Happy Mapping (Dear, P. H. and Cook, P. R. (1989) *Nucleic Acid Res. 17*:6795-6807). For these methods, the sequence of a nucleic acid fragment is used to design and produce primer pairs for use in the amplification reaction or in primer extension reactions. The design of such primers is well known to those skilled in the art. In methods employing PCR-based genetic mapping, it may be necessary to identify DNA sequence differences between the parents of the mapping cross in the region corresponding to the instant nucleic acid sequence. This, however, is generally not necessary for mapping methods.

Loss of function mutant phenotypes may be identified for the instant cDNA clones either by targeted gene disruption protocols or by identifying specific mutants for these genes contained in a maize population carrying mutations in all possible genes (Ballinger and Benzer, (1989) *Proc. Natl. Acad. Sci USA 86*:9402; Koes et al., (1995) *Proc. Natl. Acad. Sci USA 92*:8149; Bensen et al., (1995) *Plant Cell 7*:75). The latter approach may be accomplished in two ways. First, short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols in conjunction with a mutation tag sequence primer on DNAs prepared from a population of plants in which Mutator transposons or some other mutation-causing DNA element has been introduced (see Bensen, *supra*). The amplification of a specific DNA fragment with these primers indicates the insertion of the mutation tag element in or near the plant gene encoding the zeta carotene desaturase or the phytoene desaturase. Alternatively, the instant nucleic acid fragment may be used as a hybridization probe against PCR amplification products generated from the mutation population using the mutation tag sequence primer in conjunction with an arbitrary genomic site primer, such as that for a restriction enzyme site-anchored synthetic adaptor. With either method, a plant containing a mutation in the endogenous gene encoding a zeta carotene desaturase or a phytoene desaturase can be identified and obtained. This mutant plant can then be used to determine or confirm the natural function of the zeta carotene desaturase or the phytoene desaturase gene product.

EXAMPLES

The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

Composition of cDNA Libraries; Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various rice, soybean and wheat tissues were prepared. The characteristics of the libraries are described below.

TABLE 2

cDNA Libraries from Rice, Soybean and Wheat

Library	Tissue	Clone
rls6	Rice Leaf 15 Days After Germination, 6 Hours After Infection of Strain *Magaporthe grisea* 4360-R-67 (AVR2-YAMO); Susceptible	rlr6.pk0027.d5 rls6.pk0080.a9
sr1	Soybean Root	sr1.pk0022.a12
wlm12	Wheat Seedlings 12 Hours After Inoculation With *Erysiphe graminis f. sp tritici*	wlm12.pk0003.g5
wlm24	Wheat Seedlings 24 Hours After Inoculation With *Erysiphe graminis f. sp tritici*	wlm24.pk0031.e9

cDNA libraries were prepared in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). Conversion of the Uni-ZAP™ XR libraries into plasmid libraries was accomplished according to the protocol provided by Stratagene. Upon conversion, cDNA inserts were contained in the plasmid vector pBluescript. cDNA inserts from randomly picked bacterial colonies containing recombinant pBluescript plasmids were amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences or plasmid DNA was prepared from cultured bacterial cells. Amplified insert DNAs or plasmid DNAs were sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams, M. D. et al., (1991) *Science 252*:1651). The resulting ESTs were analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

EXAMPLE 2

Identification of cDNA Clones

ESTs encoding carotenoid biosynthetic enzymes were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol. 215*:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J. (1993) *Nature Genetics 3*:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the

logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

EXAMPLE 3

Characterization of cDNA Clones Encoding Zeta Carotene Desaturase

The BLASTX search using the EST sequences from clones csi1n.pk0010.f11, rls6.pk0080.a9, rlr6.pk0074.h3, sr1.pk0022.a12, sr1.pk0023.e7, wlm24.pk0031.e9 and wre1n.pk0058.h12 revealed similarity of the proteins encoded by the cDNAs to Zeta Carotene Desaturase from *Narcissus pseudonarcissus and Caspicum annum* (GenBank Accession No. AJ224683 and 1583601, respectively). Further analysis of the sequences from clones rls6.pk0080.a9 and rlr6.pk0074.h3 revealed a significant region of overalp, thus affording the assembly of a contig encoding a portion of a rice Zeta Carotene Desaturase. Likewise, analysis of the sequences from clones sr1.pk0022.a12 and sr1.pk0023.e7 revealed a significant region of overalp, thus affording the assembly of a contig encoding a portion of a soybean Zeta Carotene Desaturase. The BLAST results for each of these ESTs and contigs are shown in Table 3:

TABLE 3

BLAST Results for Clones Encoding Polypeptides Homologous to Zeta Carotene Desaturase

Clone	Organism	GenBank Accession No.	BLAST pLog Score
csi1n.pk0010.f11	*Narcissus pseudonarcissus*	AJ224683	50.30
Contig formed of: rls6.pk0080.a9 rlr6.pk0074.h3	*Caspicum annum*	1583601	40.30
Contig formed of sr1.pk0022.a12 sr1.pk0023.e7	*Caspicum annum*	1583601	10.10
wlm24.pk0031.e9	*Narcissus pseudonarcissus*	AJ224683	18.00
wre1n.pk0058.h12	*Narcissus pseudonarcissus*	AJ224683	50.70

The sequence of the entire cDNA insert in clone csi1n.pk0010.f11 was determined. BLASTP analysis indicated that it is identical to zeta carotene desaturase from *Zea mays* (NCBI General Identifier No. 4105563). The 5'-terminal sequence of the cDNA insert in clone rls6.pk0080.a9 was determined and is shown in SEQ ID NO:1; this sequence includes the partial sequence from clone rlr6.pk0074.h3. The deduced amino acid sequence of this cDNA is shown in SEQ ID NO:2. The 3'-terminal sequence of the cDNA insert in clone rls6.pk0080.a9 was determined and is shown in SEQ ID NO:3; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:4. The sequence of the entire cDNA insert in clone sr1.pk0022.a12 was determined and is shown in SEQ ID NO:7; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:8. The amino acid sequence set forth

in SEQ ID NO:8 was evaluated by BLASTP, yielding a pLog value of >254 versus the *Caspicum annum* sequence (NCBI General Identifier No. 2129927; SEQ ID NO:13). The sequence of the entire cDNA insert in clone wlm24.pk0031.e9 was determined and is shown in SEQ ID NO:7; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:8. The amino acid sequence set forth in SEQ ID NO:8 was evaluated by BLASTP, yielding a pLog value of >254 versus the *Zea mays* sequence (NCBI General Identifier No. 4105563; SEQ ID NO:14). Figure 1 presents an alignment of the amino acid sequences set forth in SEQ ID NOs:6 and 8, the *Capsicum annuum* sequence (SEQ ID NO:13) and the *Zea mays* sequence (SEQ ID NO:14). The amino acid sequence set forth in SEQ ID NO:6 is 82.9% similar to the *Capsicum annuum* sequence and the amino acid sequence set forth in SEQ ID NO:8 is 86.4% similar to the *Zea mays* sequence.

Sequence alignments and percent similarity calculations were performed using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the amino acid sequences was performed using the Clustal method of alignment (Higgins, D. G. and Sharp, P. M. (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10).

Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragments encode portions of a rice zeta carotene desaturase and entiresoybean and wheat zeta carotene desaturases. These sequences represent the first rice, soybean and wheat sequences encoding zeta carotene desaturase.

EXAMPLE 4

Characterization of cDNA Clones Encoding Phytoene Desaturase

The BLASTX search using the EST sequences from clones rlr6.pk0027.d5 and wlm12.pk0003.g5 revealed similarity of the proteins encoded by the cDNAs to Phytoene Desaturase from *Zea maize* and *Narcissus pseudonarcissus* (GenBank Accession Nos. U37285 and X78815, respectively). The BLAST results for each of these ESTs are shown in Table 4:

TABLE 4

BLAST Results for Clones Encoding Polypeptides Homologous to Phytoene Desaturase

Clone	Organism	GenBank Accession No.	BLAST pLog Score
rlr6.pk0027.d5	*Zea maize*	U37285	20.15
wlm12.pk0003.g5	*Narcissus pseudonarcissus*	X78815	29.70

The sequence of the entire cDNA insert in clone rlr6.pk0027.d5 was determined and is shown in SEQ ID NO:9; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:10. The amino acid sequence set forth in SEQ ID NO:10 was evaluated by BLASTP, yielding a pLog value of >254 versus the *Zea mays* sequence (NCBI General

Identifier No. 1345838; SEQ ID NO:15). The sequence of the entire cDNA insert in clone wlm12.pk0003.g5 was determined and is shown in SEQ ID NO:11; the deduced amino acid sequence of this cDNA is shown in SEQ ID NO:12. The amino acid sequence set forth in SEQ ID NO:12 was evaluated by BLASTP, yielding a pLog value of >254 versus the *Zea mays* sequence sequence. Figure 2 presents an alignment of the amino acid sequences set forth in SEQ ID NOs:10 and 12 and the *Zea mays* sequence (SEQ ID NO:15). The amino acid sequence set forth in SEQ ID NO:10 is 89.3% similar to the *Zea mays* sequence while the amino acid sequence set forth in SEQ ID NO:12 is 89.1% similar to the *Zea mays* sequence.

Sequence alignments and percent similarity calculations were performed using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the amino acid sequences was performed using the Clustal method of alignment (Higgins, D. G. and Sharp, P. M. (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10).

Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragments encode entire or nearly entire rice and wheat phytoene desaturases. These sequences represent the first rice and wheat sequences encoding phytoene desaturase.

EXAMPLE 5

Expression of Chimeric Genes in Monocot Cells

A chimeric gene comprising a cDNA encoding a carotenoid biosynthetic enzyme in sense orientation with respect to the maize 27 kD zein promoter that is located 5' to the cDNA fragment, and the 10 kD zein 3' end that is located 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites (Nco I or Sma I) can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the digested vector pML103 as described below. Amplification is then performed in a standard PCR. The amplified DNA is then digested with restriction enzymes Nco I and SmaI and fractionated on an agarose gel. The appropriate band can be isolated from the gel and combined with a 4.9 kb Nco I-Sma I fragment of the plasmid pML103. Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209), and bears accession number ATCC 97366. The DNA segment from pML103 contains a 1.05 kb Sal I-Nco I promoter fragment of the maize 27 kD zein gene and a 0.96 kb Sma I-Sal I fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA can be ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA may then be used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants can be screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit;

U.S. Biochemical). The resulting plasmid construct would comprise a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA fragment encoding a carotenoid biosynthetic enzyme, and the 10 kD zein 3' region.

The chimeric gene described above can then be introduced into corn cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al., (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein T. M. et al., (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1 μm in diameter) are coated with DNA using the following technique. Ten μg of plasmid DNAs are added to 50 μL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 μL of a 2.5 M solution) and spermidine free base (20 μL of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 μL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 μL of ethanol. An aliquot (5 μL) of the DNA-coated gold particles can be placed in the center of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is

then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains gluphosinate (2 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the glufosinate-supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al., (1990) *Bio/Technology 8*:833-839).

EXAMPLE 6

Expression of Chimeric Genes in Dicot Cells

A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the β subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem. 261*:9228-9238) can be used for expression of the instant carotenoid biosynthetic enzyme in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites Nco I (which includes the ATG translation initiation codon), Sma I, Kpn I and Xba I. The entire cassette is flanked by Hind III sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embroys may then be transformed with the expression vector comprising sequences encoding a carotenoid biosynthetic enzyme. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein T. M. et al. (1987) *Nature* (London) *327*:70-73, U.S. Patent No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al.(1983) *Gene 25*:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression cassette comprising the phaseolin 5' region, the fragment encoding the carotenoid biosynthetic enzyme and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µL of a 60 mg/mL 1 µm gold particle suspension is added (in order): 5 µL DNA (1 µg/µL), 20 µl spermidine (0.1 M), and 50 µL $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µL 70% ethanol and resuspended in 40 µL of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five µL of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These

suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 7

Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant carotenoid biosynthetic enzyme can be inserted into the T7 *E. coli* expression vector pBT430. This vector is a derivative of pET-3a (Rosenberg et al. (1987) *Gene 56*:125-135) which employs the bacteriophage T7 RNA polymerase/T7 promoter system. Plasmid pBT430 was constructed by first destroying the EcoR I and Hind III sites in pET-3a at their original positions. An oligonucleotide adaptor containing EcoR I and Hind III sites was inserted at the BamH I site of pET-3a. This created pET-3aM with additional unique cloning sites for insertion of genes into the expression vector. Then, the Nde I site at the position of translation initiation was converted to an Nco I site using oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region, 5'-CATATGG, was converted to 5'-CCCATGG in pBT430.

Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the protein. This fragment may then be purified on a 1% NuSieve GTG™ low melting agarose gel (FMC). Buffer and agarose contain 10 μg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELase™ (Epicentre Technologies) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 μL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs, Beverly, MA). The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as described above. The prepared vector pBT430 and fragment can then be ligated at 16°C for 15 hours followed by transformation into DH5 electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and 100 μg/mL ampicillin. Transformants containing the gene encoding the carotenoid biosynthetic enzyme are then screened for the correct orientation with respect to the T7 promoter by restriction enzyme analysis.

For high level expression, a plasmid clone with the cDNA insert in the correct orientation relative to the T7 promoter can be transformed into *E. coli* strain BL21(DE3) (Studier et al. (1986) *J. Mol. Biol. 189*:113-130). Cultures are grown in LB medium containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately 1, IPTG (isopropylthio-β-galactoside, the inducer) can be added to a final concentration of 0.4 mM and incubation can be continued for 3 h at 25°. Cells are then harvested by centrifugation and re-suspended in 50 μL of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe

sonicator. The mixture is centrifuged and the protein concentration of the supernatant determined. One μg of protein from the soluble fraction of the culture can be separated by SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating at the expected molecular weight.

EXAMPLE 8

Evaluating Compounds for Their Ability to Inhibit the Activity of Carotenoid Biosynthetic Enzymes

The carotenoid biosynthetic enzymes described herein may be produced using any number of methods known to those skilled in the art. Such methods include, but are not limited to, expression in bacteria as described in Example 7, or expression in eukaryotic cell culture, *in planta*, and using viral expression systems in suitably infected organisms or cell lines. The instant carotenoid biosynthetic enzymes may be expressed either as mature forms of the proteins as observed *in vivo* or as fusion proteins by covalent attachment to a variety of enzymes, proteins or affinity tags. Common fusion protein partners include glutathione S-transferase ("GST"), thioredoxin ("Trx"), maltose binding protein, and C- and/or N-terminal hexahistidine polypeptide ("$(His)_6$"). The fusion proteins may be engineered with a protease recognition site at the fusion point so that fusion partners can be separated by protease digestion to yield intact mature enzyme. Examples of such proteases include thrombin, enterokinase and factor Xa. However, any protease can be used which specifically cleaves the peptide connecting the fusion protein and the enzyme.

Purification of the instant carotenoid biosynthetic enzymes, if desired, may utilize any number of separation technologies familiar to those skilled in the art of protein purification. Examples of such methods include, but are not limited to, homogenization, filtration, centrifugation, heat denaturation, ammonium sulfate precipitation, desalting, pH precipitation, ion exchange chromatography, hydrophobic interaction chromatography and affinity chromatography, wherein the affinity ligand represents a substrate, substrate analog or inhibitor. When the carotenoid biosynthetic enzymes are expressed as fusion proteins, the purification protocol may include the use of an affinity resin which is specific for the fusion protein tag attached to the expressed enzyme or an affinity resin containing ligands which are specific for the enzyme. For example, a carotenoid biosynthetic enzyme may be expressed as a fusion protein coupled to the C-terminus of thioredoxin. In addition, a $(His)_6$ peptide may be engineered into the N-terminus of the fused thioredoxin moiety to afford additional opportunities for affinity purification. Other suitable affinity resins could be synthesized by linking the appropriate ligands to any suitable resin such as Sepharose-4B. In an alternate embodiment, a thioredoxin fusion protein may be eluted using dithiothreitol; however, elution may be accomplished using other reagents which interact to displace the thioredoxin from the resin. These reagents include β–mercaptoethanol or other reduced thiol. The eluted fusion protein may be subjected to further purification by traditional means as stated above, if desired. Proteolytic cleavage of the thioredoxin fusion protein and the

enzyme may be accomplished after the fusion protein is purified or while the protein is still bound to the ThioBond™ affinity resin or other resin.

Crude, partially purified or purified enzyme, either alone or as a fusion protein, may be utilized in assays for the evaluation of compounds for their ability to inhibit enzymatic activation of the carotenoid biosynthetic enzymes disclosed herein. Assays may be conducted under well known experimental conditions which permit optimal enzymatic activity. For example, assays for zeta carotene desaturase are presented by Linden H. et al. (1994) *Plant Mol. Biol. 24*:369-379. Assays for phytoene desaturase are presented by Pecker I. et al. (1992) *Proc Natl Acad Sci U S A 89*:4962-4966.

CLAIMS

What is claimed is:

1. An isolated nucleic acid fragment encoding all or a substantial portion of a zeta carotene desaturase comprising a member selected from the group consisting of:

(a) an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8;

(b) an isolated nucleic acid fragment that is substantially similar to an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8; and

(c) an isolated nucleic acid fragment that is complementary to (a) or (b).

2. The isolated nucleic acid fragment of Claim 1 wherein the nucleotide sequence of the fragment comprises all or a portion of the sequence set forth in a member selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7.

3. A chimeric gene comprising the nucleic acid fragment of Claim 1 operably linked to suitable regulatory sequences.

4. A transformed host cell comprising the chimeric gene of Claim 3.

5. A zeta carotene desaturase polypeptide comprising all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8.

6. An isolated nucleic acid fragment encoding all or a substantial portion of a phytoene desaturase comprising a member selected from the group consisting of:

(a) an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:12;

(b) an isolated nucleic acid fragment that is substantially similar to an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:12; and

(c) an isolated nucleic acid fragment that is complementary to (a) or (b).

7. The isolated nucleic acid fragment of Claim 6 wherein the nucleotide sequence of the fragment comprises all or a portion of the sequence set forth in a member selected from the group consisting of SEQ ID NO:9 and SEQ ID NO:11.

8. A chimeric gene comprising the nucleic acid fragment of Claim 6 operably linked to suitable regulatory sequences.

9. A transformed host cell comprising the chimeric gene of Claim 8.

10. A phytoene desaturase polypeptide comprising all or a substantial portion of the amino acid sequence set forth in a member selected from the group consisting of SEQ ID NO:10 and SEQ ID NO:12.

11. A method of altering the level of expression of a carotenoid biosynthetic enzyme in a host cell comprising:

(a) transforming a host cell with the chimeric gene of any of Claims 3 and 8; and

(b) growing the transformed host cell produced in step (a) under conditions that are suitable for expression of the chimeric gene

wherein expression of the chimeric gene results in production of altered levels of a carotenoid biosynthetic enzyme in the transformed host cell.

12. A method of obtaining a nucleic acid fragment encoding all or a substantial portion of the amino acid sequence encoding a carotenoid biosynthetic enzyme comprising:

(a) probing a cDNA or genomic library with the nucleic acid fragment of any of Claims 1 and 6;

(b) identifying a DNA clone that hybridizes with the nucleic acid fragment of any of Claims 1and 6;

(c) isolating the DNA clone identified in step (b); and

(d) sequencing the cDNA or genomic fragment that comprises the clone isolated in step (c)

wherein the sequenced nucleic acid fragment encodes all or a substantial portion of the amino acid sequence encoding a carotenoid biosynthetic enzyme.

13. A method of obtaining a nucleic acid fragment encoding a substantial portion of an amino acid sequence encoding a carotenoid biosynthetic enzyme comprising:

(a) synthesizing an oligonucleotide primer corresponding to a portion of the sequence set forth in any of SEQ ID NOs:1, 3, 5, 7, 9 and 11; and

(b) amplifying a cDNA insert present in a cloning vector using the oligonucleotide primer of step (a) and a primer representing sequences of the cloning vector

wherein the amplified nucleic acid fragment encodes a substantial portion of an amino acid sequence encoding a carotenoid biosynthetic enzyme.

14. The product of the method of Claim 12.

15. The product of the method of Claim 13.

16. A method for evaluating at least one compound for its ability to inhibit the activity of a carotenoid biosynthetic enzyme, the method comprising the steps of:

(a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a carotenoid biosynthetic enzyme, operably linked to suitable regulatory sequences;

(b) growing the transformed host cell under conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of the carotenoid biosynthetic enzyme encoded by the operably linked nucleic acid fragment in the transformed host cell;

(c) optionally purifying the carotenoid biosynthetic enzyme expressed by the transformed host cell;

(d) treating the carotenoid biosynthetic enzyme with a compound to be tested; and

(e) comparing the activity of the carotenoid biosynthetic enzyme that has been treated with a test compound to the activity of an untreated carotenoid biosynthetic enzyme,

thereby selecting compounds with potential for inhibitory activity.

FIGURE 1

```
                **                                                *         *
SEQ ID NO:13    MASVAA--TTTLAPALAPRRARPGTG---------LVPPR--RASAVAARSTVTSPTWR-
SEQ ID NO:14    MATCSAYLCCPATSASLKKRVFPDGSAGFLFFGGRRLSNRLV-TPKSVIRADLNSMVSDM
SEQ ID NO:06    MAS---LIQCSATS-----------------LSAVPITTRFTRTHKSRLRCSLDANVSDM
SEQ ID NO:08    MA--AT--SCALVSALVVGRRR---G---------PSCQR--AAAAGVVRCSLDSKVSDM
                1                                                           60

                ****************************************** * ** *******
SEQ ID NO:13    --QRSQRLFPPEPEHYRGPKLKVAIIGAGLAGMSTAVELLDQGHEVDLYESRPFIGGKVG
SEQ ID NO:14    STNAPKGLFPPEPEHYRGPKLKVAIIGAGLAGMSTAVELLDQGHEVDIYESRTFIGGKVG
SEQ ID NO:06    SVNAPKGLFPPEPEHYRGPKLKVAIIGAGLAGMSTAVELLDQGHEVDIYESRPFIGGKVG
SEQ ID NO:08    AINAPKGLFPPEPEHYRGPKLKVAIIGAGLAGMSTAVELLDQGHEVDLYDSRTFIGGKVG
                61                                                          120

                ****  *************** ***** *****  ***** **********  *******
SEQ ID NO:13    SFVDRQGNHIEMGLHVFFGCYSNLFRLMKKVGADNNLLVKEHTHTFVNKGGTIGELDFRF
SEQ ID NO:14    SFVDKRGNHIEMGLHVFFGCYNNLFRLMKKVGAEKNLLVKEHTHTFVNKGGEIGELDFRF
SEQ ID NO:06    SFVDKGGNHIEMGLHVFFGCYNNLFRLLKKVGAENNLLVKDHTHTFVNKGGQIGELDFRF
SEQ ID NO:08    SFVDKHGNHIEMGLHVFFGCYSNLFRLMKKVGADNNLLVKEHTHTFVNKGGIVGELDFRF
                121                                                         180

                ********* *** ****  ******************* ******   * **  ** **
SEQ ID NO:13    PVGAPLHGIQAFLRTNQLKVYDKARNAVALALSPVVRALVDPDGALQQVRDLDDISFSDW
SEQ ID NO:14    PVGAPLHGINAFLSTNQLKTYDKARNAVALALSPVVRALVDPDGALQQIRDLDSVSFSDW
SEQ ID NO:06    PIGAPIHGIRAFLTTNQLNTYDKARNAVALALSPVVRALVDPDGALRDIRNLDSISFSDW
SEQ ID NO:08    PVGAPLHGIQAFLRTNQLKVYDKARNAVALALSPVVRALLDPDGALQQVRDLDDVSFTDW
                181                                                         240
```

FIGURE 1 - CONTINUED

```
                * * **** ** ****** ********************* ********************
SEQ ID NO:13    FMSKGGTRESITRMWDPVRYALGFIDCDNISARCMLTIFTLFATKTEASLLRMLKGSPDV
SEQ ID NO:14    FMSKGGTRASIQRMWDPVAYALGFIDCDNISARCMLTIFALFATKTEASLLRMLKGSPDV
SEQ ID NO:06    FLSKGGTRMSITKMWDPVAYALGFIDCDNISARCMLTIFALFATKTEASLLRMLKGSPDV
SEQ ID NO:08    FMSRGGTRESITRMWDPVAYALGFIDCDNISARCMLTIFTLFATKTEASLLRMLKGSPDV
                241                                                     300

                ****** *** * ************ **  * **  ** *    ***   * ********
SEQ ID NO:13    YLSGPIKKYITDRGGRFHLRWGCREVLYEKSPDGETYVKGLLLTKATSREIIKADAYVAA
SEQ ID NO:14    YLSGPIKKYIIDKGGRFHLRWGCREVLYETSSDGSMYVSGLAMSKATQKKIVKADAYVAA
SEQ ID NO:06    YLSGPIRKYIMDRGGRFHLRWGCRELLYDKSADGSIYVTGLSMSKATAKKIVKADAYVAA
SEQ ID NO:08    YLSGPIKKYITDRGGRFHLKWGCREVLYDKSPDGETYVKGFLISKATSSEIIKADAYVAA
                301                                                     360

                ********* *  ***   * *** * ***************** **** **    * **
SEQ ID NO:13    CDVPGIKRLLPSEWREWEMFDNIYKLDGVPVVTVQLRYNGWVTELQDLEKSRQLQRAVGL
SEQ ID NO:14    CVVPGIKRLVPQKWRELEFFGNIYKLIGVPVVTVQLRYNGWVTELQDLERSRQSKRATGL
SEQ ID NO:06    CDVPGIKRLLPSEWREQEFFNNIYELVGVPVVTVQLRYNGWVTELQDLEKSRRLGKAVGL
SEQ ID NO:08    CDVPGIKRLLPSEWREWDMFDNIYKLDGVPVVTVQLRYNGWVTEVQDLEKSRQLQKAVGL
                361                                                     420

                ****** ******* **** ** ********** * ************ ***  * ***
SEQ ID NO:13    DNLLYTADADFSCFSDLALSSPADYYIEGQGSLIQAVLTPGDPYMPLPNEEIISKVQKQV
SEQ ID NO:14    DNLLYTPDADFSCFADLALASPEDYYIEGQGSLLQCVLTPGDPYMPLPNEEIIRRVSKQV
SEQ ID NO:06    DNLLYTPDADFSCFADLALSSPEDYYIEGQGSLLQCVLTPGDPYMPLPNDEIIARVAKQV
SEQ ID NO:08    DNLLYTPDADFSCFSDLALSSPADYYIEGQGSLIQAVLTPGDPYMPLPNEEIISKVEKQV
                421                                                     480
```

FIGURE 1 - CONTINUED

```
                 ****  ******************** ** ** ******** **** ************
SEQ ID NO:13     VELFPSSRGLEVTWSSVVKIGQSLYREAPGNDPFRPDQKTPVKNFFLSGSYTKQDYIDSM
SEQ ID NO:14     LALFPSSQGLEVTWSSVVKIGQSLYREGPGKDPFRPDQKTPVENFFLAGSYTKQDYIDSM
SEQ ID NO:06     LALFPSSQGLEVTWSSVVKIGQSLYREGPGKDPYRPDQKTPVRNFFLAGSYTKQDYIDSM
SEQ ID NO:08     LDLFPSARGLEVTWSSVVKIGQSLYREAPGNDPFRPDQKTPVKNFFLSGSYTKQDYIDSM
                 481                                                       540

                 ********   **** *** * * ***          *       *
SEQ ID NO:13     EGATLSGRRTSAYICGAGEELLALRKKLL---IDDGEKALGNVQVLQAS
SEQ ID NO:14     EGATLSGRQASAYICDAGEQLLALRKKIAAAELNEISKGVSLSDELSLV
SEQ ID NO:06     EGATLSGRQASAYICDAGEELVALRKKLDA-EFKDDLKISNTKDELSLV
SEQ ID NO:08     EGATLSGRRTAAYICGAGEELLAIRKKLI---VDHSEKASGMVQMLQTS
                 541                                              589
```

FIGURE 2

```
SEQ ID NO:15  MDTGCLSSMNITGASQTRSFAGQLPPQRCFASS--------HYTSFAVKKLVSRNKGRRS
SEQ ID NO:10  MDTGCLSSMNITGTSQARSFAGQLPTHRCFASSSIQALKSSQHVSFGVKSLVLRNKGKRF
SEQ ID NO:12  ------------------------------------------------------------
              1                                                          60

SEQ ID NO:15  HRRHPALQVVCKDFPRPPLESTINYLEAGQLSSFFRNSERPSKPLQVVVAGAGLAGLSTA
SEQ ID NO:10  RRRLGALQVVCQDFPRPPLENTINFLEAGQLSSFFRNSEQPTKPLQVVIAGAGLAGLSTA
SEQ ID NO:12  ------------------------------------------------------------
              61                                                         120

                                                        * ** * **
SEQ ID NO:15  KYLADAGHKPILLEARDVLGGKVAAWKDEDGDWYETGLHIFFGAYPNIQNLFGELRIEDR
SEQ ID NO:10  KYLADAGHKPILLEARDVLGGKIAAWKDEDGDWYETGLHIFFGAYPNIQNLFGELGINDR
SEQ ID NO:12  ------------------------------------------TRFAELGISDR
              121                                                        180

              ****************** ************ ** **** ************** *****
SEQ ID NO:15  LQWKEHSMIFAMPNKPGEFSRFDFPETLPAPINGIWAILRNNEMLTWPEKVKFAIGLLPA
SEQ ID NO:10  LQWKEHSMIFAMPNKPGEFSRFDFPETLPAPLNGIWAILRNNEMLTWPEKVKFALGLLPA
SEQ ID NO:12  LQWKEHSMIFAMPNKPGEYSRFDFPETLPAPLNGVWAILKNNEMLTWPEKVKFAIGLLPA
              181                                                        240
```

FIGURE 2 -CONTINUED

```
                * *** ******* ****** ****************************************
SEQ ID NO:15    MVGGQPYVEAQDGLTVSEWMKKQGVPDRVNDEVFIAMSKALNFINPDELSMQCILIALNR
SEQ ID NO:10    MVGGQAYVEAQDGFTVSEWMKKQGVPDRVNDEVFIAMSKALNFINPDELSMQCILIALNR
SEQ ID NO:12    MLGGQAYVEAQDGLTVSEWMEKQGVPDRVNDEVFIAMSKALNFINPDELSMQCILIALNR
                241                                                       300

                *************************** *  * ********** *************** *
SEQ ID NO:15    FLQEKHGSKMAFLDGNPPERLCMPIVDHIRSRGGEVRLNSRIKKIELNPDGTVKHFALSD
SEQ ID NO:10    FLQEKHGSKMAFLDGNPPERLCMPIVDHVRSLGGEVRLNSRIQKIELNPDGTVKHFALTD
SEQ ID NO:12    FLQEKHGSKMAFLDGNPPERLCMPIVNHIQSLGGEVRLNSRIQKIELNPDGTVKHFALTD
                301                                                       360

                ********** * **** ******** ** *** * ****************** *****
SEQ ID NO:15    GTQITGDAYVCATPVDIFKLLVPQEWSEITYFKKLEKLVGVPVINVHIWFDRKLNNTYDH
SEQ ID NO:10    GTQITGDAYVFATPVDILKLLVPQEWKEISYFKKLEKLVGVPVINVHIWFDRKLKNTYDH
SEQ ID NO:12    GTQITGDAYVFAAPVDIFKLLVPQEWREISYFKRLDKLVGVPVINVHIWFDRKLKNTYDH
                361                                                       420

                ****************  ******  ********** ** ******** *** ******
SEQ ID NO:15    LLFSRSSLLSVYADMSVTCKEYYDPNRSMLELVFAPADEWIGRSDTEIIDATMEELAKLF
SEQ ID NO:10    LLFSRSSLLSVYADMSVTCKEYYDQKRSMLELVFAPAEEWVGRSDTEIIEATMQELAKLF
SEQ ID NO:12    LLFSRSSLLSVYADMSLACKEYYDPNRSMLELVFAPAEEWIGRSDTEIIEATMLELAKLF
                421                                                       480
```

FIGURE 2 - CONTINUED

```
                  ****************** ********** * ******************************
SEQ ID NO:15      PDEIAADQSKAKILKYHIVKTPRSVYKTVPNCEPCRPLQRSPIEGFYLAGDYTKQKYLAS
SEQ ID NO:10      PDEIAADQSKAKILKYHVVKTPRSVYKTIPDCEPCRPLQRSPIEGFYLAGDYTKQKYLAS
SEQ ID NO:12      PDEIAADQSKAKILKYHVVKTPRSVYKTVPNCEPCRPLQRSPIEGFYLAGDYTKQKYLAS
                  481                                                       540

                  ********* **** * *   *  **  **** * **
SEQ ID NO:15      MEGAVLSGKLCAQSIVQDYSRLALRSQKSLQSGEVPVP---S
SEQ ID NO:10      MEGAVLSGKLCAQSVVEDYKMLSRRSLKSLQS-EVPVAS.--
SEQ ID NO:12      MEGAVLSGKFCAQSIVQDSKMLSRRSQESLQS-EAPVASKL.
                  541                                      582
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SEQUENCE LISTING

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ccatttatac tctcctcctc ctcctcctcc tctactccta gcagcagcgt tggttggtga 180
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35 40 45

Val Ser Asp Met Ala Val Asn Ala Pro Lys Gly Leu Phe Pro Pro Glu
50 55 60

Pro Glu His Tyr Arg Gly Pro Lys Leu Lys Val Ala Ile Ile Trp Ala
65 70 75 80

Gly Leu Ala Gly Met Ser Thr Ala Val Glu Leu Leu Asp Gln Cys His
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20 25 30

Xaa Glu Leu Phe Pro Ser Ser Gln Gly Leu Glu Leu Thr Trp Ser Ser
35 40 45

Leu Val Lys Ile Gly Gln Ser Leu Tyr Arg Glu Ser Pro Gly Ile Asn
50 55 60

Pro Phe Arg Pro Asp Gln Lys Thr Pro Val Lys Asn Phe Phe Leu Ser
65 70 75 80

Gly Ser Tyr Thr Lys Gln Asp Tyr Ile Asp Ser Met Glu Gly Ala Thr
85 90 95

Leu Ser Gly Arg Arg Thr Ala Ala Tyr Ile Cys Gly Ala Gly Glu Glu
100 105 110

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115 120 125

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35 40 45

Phe Pro Pro Glu Pro Glu His Tyr Arg Gly Pro Lys Leu Lys Val Ala
50 55 60

Ile Ile Gly Ala Gly Leu Ala Gly Met Ser Thr Ala Val Glu Leu Leu
65 70 75 80

Asp Gln Gly His Glu Val Asp Ile Tyr Glu Ser Arg Pro Phe Ile Gly
85 90 95

Gly Lys Val Gly Ser Phe Val Asp Lys Gly Gly Asn His Ile Glu Met
100 105 110

Gly Leu His Val Phe Phe Gly Cys Tyr Asn Asn Leu Phe Arg Leu Leu
115 120 125

Lys Lys Val Gly Ala Glu Asn Asn Leu Leu Val Lys Asp His Thr His
130 135 140

Thr Phe Val Asn Lys Gly Gly Gln Ile Gly Glu Leu Asp Phe Arg Phe
145 150 155 160

Pro Ile Gly Ala Pro Ile His Gly Ile Arg Ala Phe Leu Thr Thr Asn
165 170 175

Gln Leu Asn Thr Tyr Asp Lys Ala Arg Asn Ala Val Ala Leu Ala Leu
180 185 190

Ser Pro Val Val Arg Ala Leu Val Asp Pro Asp Gly Ala Leu Arg Asp
195 200 205

Ile Arg Asn Leu Asp Ser Ile Ser Phe Ser Asp Trp Phe Leu Ser Lys
210 215 220

Gly Gly Thr Arg Met Ser Ile Thr Lys Met Trp Asp Pro Val Ala Tyr
225 230 235 240

Ala Leu Gly Phe Ile Asp Cys Asp Asn Ile Ser Ala Arg Cys Met Leu
245 250 255

Thr Ile Phe Ala Leu Phe Ala Thr Lys Thr Glu Ala Ser Leu Leu Arg
260 265 270

Met Leu Lys Gly Ser Pro Asp Val Tyr Leu Ser Gly Pro Ile Arg Lys
275 280 285

Tyr Ile Met Asp Arg Gly Gly Arg Phe His Leu Arg Trp Gly Cys Arg
290 295 300

Glu Leu Leu Tyr Asp Lys Ser Ala Asp Gly Ser Ile Tyr Val Thr Gly
305 310 315 320

Leu Ser Met Ser Lys Ala Thr Ala Lys Lys Ile Val Lys Ala Asp Ala
325 330 335

Tyr Val Ala Ala Cys Asp Val Pro Gly Ile Lys Arg Leu Leu Pro Ser
340 345 350

Glu Trp Arg Glu Gln Glu Phe Phe Asn Asn Ile Tyr Glu Leu Val Gly
355 360 365

Val Pro Val Val Thr Val Gln Leu Arg Tyr Asn Gly Trp Val Thr Glu
370 375 380

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Leu Gln Asp Leu Glu Lys Ser Arg Arg Leu Gly Lys Ala Val Gly Leu
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Asp Asn Leu Leu Tyr Thr Pro Asp Ala Asp Phe Ser Cys Phe Ala Asp
                405                 410                 415

Leu Ala Leu Ser Ser Pro Glu Asp Tyr Tyr Ile Glu Gly Gln Gly Ser
            420                 425                 430

Leu Leu Gln Cys Val Leu Thr Pro Gly Asp Pro Tyr Met Pro Leu Pro
        435                 440                 445

Asn Asp Glu Ile Ile Ala Arg Val Ala Lys Gln Val Leu Ala Leu Phe
    450                 455                 460

Pro Ser Ser Gln Gly Leu Glu Val Thr Trp Ser Ser Val Val Lys Ile
465                 470                 475                 480

Gly Gln Ser Leu Tyr Arg Glu Gly Pro Gly Lys Asp Pro Tyr Arg Pro
                485                 490                 495

Asp Gln Lys Thr Pro Val Arg Asn Phe Phe Leu Ala Gly Ser Tyr Thr
            500                 505                 510

Lys Gln Asp Tyr Ile Asp Ser Met Glu Gly Ala Thr Leu Ser Gly Arg
        515                 520                 525

Gln Ala Ser Ala Tyr Ile Cys Asp Ala Gly Glu Glu Leu Val Ala Leu
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Arg Lys Lys Leu Asp Ala Glu Phe Lys Asp Asp Leu Lys Ile Ser Asn
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Thr Lys Asp Glu Leu Ser Leu Val
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<213> Triticum aestivum

<400> 7

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<213> Triticum aestivum

<400> 8

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Cys Ser Leu Asp Ser Lys Val Ser Asp Met Ala Ile Asn Ala Pro Lys
         35                  40                  45

Gly Leu Phe Pro Pro Glu Pro Glu His Tyr Arg Gly Pro Lys Leu Lys
     50                  55                  60

Val Ala Ile Ile Gly Ala Gly Leu Ala Gly Met Ser Thr Ala Val Glu
 65                  70                  75                  80

Leu Leu Asp Gln Gly His Glu Val Asp Leu Tyr Asp Ser Arg Thr Phe
                 85                  90                  95

Ile Gly Gly Lys Val Gly Ser Phe Val Asp Lys His Gly Asn His Ile
            100                 105                 110

Glu Met Gly Leu His Val Phe Phe Gly Cys Tyr Ser Asn Leu Phe Arg
        115                 120                 125

Leu Met Lys Lys Val Gly Ala Asp Asn Asn Leu Leu Val Lys Glu His
    130                 135                 140

Thr His Thr Phe Val Asn Lys Gly Gly Ile Val Gly Glu Leu Asp Phe
145                 150                 155                 160

Arg Phe Pro Val Gly Ala Pro Leu His Gly Ile Gln Ala Phe Leu Arg
                165                 170                 175

Thr Asn Gln Leu Lys Val Tyr Asp Lys Ala Arg Asn Ala Val Ala Leu
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Ala Leu Ser Pro Val Val Arg Ala Leu Leu Asp Pro Asp Gly Ala Leu
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Gln Gln Val Arg Asp Leu Asp Asp Val Ser Phe Thr Asp Trp Phe Met
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Ser Arg Gly Gly Thr Arg Glu Ser Ile Thr Arg Met Trp Asp Pro Val
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Ala Tyr Ala Leu Gly Phe Ile Asp Cys Asp Asn Ile Ser Ala Arg Cys
                245                 250                 255

Met Leu Thr Ile Phe Thr Leu Phe Ala Thr Lys Thr Glu Ala Ser Leu
            260                 265                 270

Leu Arg Met Leu Lys Gly Ser Pro Asp Val Tyr Leu Ser Gly Pro Ile
        275                 280                 285

Lys Lys Tyr Ile Thr Asp Arg Gly Gly Arg Phe His Leu Lys Trp Gly
    290                 295                 300

Cys Arg Glu Val Leu Tyr Asp Lys Ser Pro Asp Gly Glu Thr Tyr Val
305                 310                 315                 320

Lys Gly Phe Leu Ile Ser Lys Ala Thr Ser Ser Glu Ile Ile Lys Ala
                325                 330                 335

Asp Ala Tyr Val Ala Ala Cys Asp Val Pro Gly Ile Lys Arg Leu Leu
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Pro Ser Glu Trp Arg Glu Trp Asp Met Phe Asp Asn Ile Tyr Lys Leu
        355                 360                 365

Asp Gly Val Pro Val Val Thr Val Gln Leu Arg Tyr Asn Gly Trp Val
    370                 375                 380

Thr Glu Val Gln Asp Leu Glu Lys Ser Arg Gln Leu Gln Lys Ala Val
385                 390                 395                 400

Gly Leu Asp Asn Leu Leu Tyr Thr Pro Asp Ala Asp Phe Ser Cys Phe
                405                 410                 415

Ser Asp Leu Ala Leu Ser Ser Pro Ala Asp Tyr Tyr Ile Glu Gly Gln
            420                 425                 430

Gly Ser Leu Ile Gln Ala Val Leu Thr Pro Gly Asp Pro Tyr Met Pro
        435                 440                 445

Leu Pro Asn Glu Glu Ile Ile Ser Lys Val Glu Lys Gln Val Leu Asp
    450                 455                 460

Leu Phe Pro Ser Ala Arg Gly Leu Glu Val Thr Trp Ser Ser Val Val
465                 470                 475                 480

Lys Ile Gly Gln Ser Leu Tyr Arg Glu Ala Pro Gly Asn Asp Pro Phe
                485                 490                 495

Arg Pro Asp Gln Lys Thr Pro Val Lys Asn Phe Phe Leu Ser Gly Ser
            500                 505                 510

Tyr Thr Lys Gln Asp Tyr Ile Asp Ser Met Glu Gly Ala Thr Leu Ser
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Gly Arg Arg Thr Ala Ala Tyr Ile Cys Gly Ala Gly Glu Glu Leu Leu
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Ala Ile Arg Lys Lys Leu Ile Val Asp His Ser Glu Lys Ala Ser Gly
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Met Val Gln Met Leu Gln Thr Ser
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<212> DNA
<213> Oryza sativa

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<400> 10

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Ser Ser Ile Gln Ala Leu Lys Ser Ser Gln His Val Ser Phe Gly Val
        35                  40                  45

Lys Ser Leu Val Leu Arg Asn Lys Gly Lys Arg Phe Arg Arg Arg Leu
    50                  55                  60

Gly Ala Leu Gln Val Val Cys Gln Asp Phe Pro Arg Pro Pro Leu Glu
65                  70                  75                  80

Asn Thr Ile Asn Phe Leu Glu Ala Gly Gln Leu Ser Ser Phe Phe Arg
                85                  90                  95

Asn Ser Glu Gln Pro Thr Lys Pro Leu Gln Val Val Ile Ala Gly Ala
            100                 105                 110

Gly Leu Ala Gly Leu Ser Thr Ala Lys Tyr Leu Ala Asp Ala Gly His
        115                 120                 125

Lys Pro Ile Leu Leu Glu Ala Arg Asp Val Leu Gly Gly Lys Ile Ala
    130                 135                 140

Ala Trp Lys Asp Glu Asp Gly Asp Trp Tyr Glu Thr Gly Leu His Ile
145                 150                 155                 160

Phe Phe Gly Ala Tyr Pro Asn Ile Gln Asn Leu Phe Gly Glu Leu Gly
                165                 170                 175

Ile Asn Asp Arg Leu Gln Trp Lys Glu His Ser Met Ile Phe Ala Met
            180                 185                 190

Pro Asn Lys Pro Gly Glu Phe Ser Arg Phe Asp Phe Pro Glu Thr Leu
        195                 200                 205

Pro Ala Pro Leu Asn Gly Ile Trp Ala Ile Leu Arg Asn Asn Glu Met
    210                 215                 220

Leu Thr Trp Pro Glu Lys Val Lys Phe Ala Leu Gly Leu Leu Pro Ala
225                 230                 235                 240

Met Val Gly Gly Gln Ala Tyr Val Glu Ala Gln Asp Gly Phe Thr Val
                245                 250                 255

Ser Glu Trp Met Lys Lys Gln Gly Val Pro Asp Arg Val Asn Asp Glu
            260                 265                 270

Val Phe Ile Ala Met Ser Lys Ala Leu Asn Phe Ile Asn Pro Asp Glu
        275                 280                 285

Leu Ser Met Gln Cys Ile Leu Ile Ala Leu Asn Arg Phe Leu Gln Glu
    290                 295                 300

Lys His Gly Ser Lys Met Ala Phe Leu Asp Gly Asn Pro Pro Glu Arg
305                 310                 315                 320
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Leu Cys Met Pro Ile Val Asp His Val Arg Ser Leu Gly Gly Glu Val
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Arg Leu Asn Ser Arg Ile Gln Lys Ile Glu Leu Asn Pro Asp Gly Thr
            340                 345                 350

Val Lys His Phe Ala Leu Thr Asp Gly Thr Gln Ile Thr Gly Asp Ala
        355                 360                 365

Tyr Val Phe Ala Thr Pro Val Asp Ile Leu Lys Leu Leu Val Pro Gln
    370                 375                 380

Glu Trp Lys Glu Ile Ser Tyr Phe Lys Lys Leu Glu Lys Leu Val Gly
385                 390                 395                 400

Val Pro Val Ile Asn Val His Ile Trp Phe Asp Arg Lys Leu Lys Asn
                405                 410                 415

Thr Tyr Asp His Leu Leu Phe Ser Arg Ser Ser Leu Leu Ser Val Tyr
            420                 425                 430

Ala Asp Met Ser Val Thr Cys Lys Glu Tyr Tyr Asp Gln Lys Arg Ser
        435                 440                 445

Met Leu Glu Leu Val Phe Ala Pro Ala Glu Glu Trp Val Gly Arg Ser
    450                 455                 460

Asp Thr Glu Ile Ile Glu Ala Thr Met Gln Glu Leu Ala Lys Leu Phe
465                 470                 475                 480

Pro Asp Glu Ile Ala Ala Asp Gln Ser Lys Ala Lys Ile Leu Lys Tyr
                485                 490                 495

His Val Val Lys Thr Pro Arg Ser Val Tyr Lys Thr Ile Pro Asp Cys
            500                 505                 510

Glu Pro Cys Arg Pro Leu Gln Arg Ser Pro Ile Glu Gly Phe Tyr Leu
        515                 520                 525

Ala Gly Asp Tyr Thr Lys Gln Lys Tyr Leu Ala Ser Met Glu Gly Ala
    530                 535                 540

Val Leu Ser Gly Lys Leu Cys Ala Gln Ser Val Val Glu Asp Tyr Lys
545                 550                 555                 560

Met Leu Ser Arg Arg Ser Leu Lys Ser Leu Gln Ser Glu Val Pro Val
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Ala Ser
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Glu Leu Leu Asp Gln Gly His Glu Val Asp Ile Tyr Glu Ser Arg Thr
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225                 230                 235                 240

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Glu His Ser Met Ile Phe Ala Met Pro Asn Lys Pro Gly Glu Phe Ser
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Arg Phe Asp Phe Pro Glu Thr Leu Pro Ala Pro Ile Asn Gly Ile Trp
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Ala Ile Leu Arg Asn Asn Glu Met Leu Thr Trp Pro Glu Lys Val Lys
    210                 215                 220

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225                 230                 235                 240

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                245                 250                 255

Val Pro Asp Arg Val Asn Asp Glu Val Phe Ile Ala Met Ser Lys Ala
            260                 265                 270

Leu Asn Phe Ile Asn Pro Asp Glu Leu Ser Met Gln Cys Ile Leu Ile
        275                 280                 285

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    290                 295                 300

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C12N 15/82, 9/02, 5/10, G01N 33/50
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A3

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(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CAHOON, Rebecca, E. [US/US]; 2331 West 18th Street, Wilmington, DE 19806 (US). KINNEY, Anthony, J. [GB/US]; 609 Lore Avenue, Wilmington, DE 19809 (US). VOLLMER, Steven, J. [US/US]; 1019 Tweedbrook Road, Wilmington, DE 19810 (US). WENG, Zude [CN/US]; 909C Cloister Road, Wilmington, DE 19809 (US).

(74) Agent: MAJARIAN, William, R.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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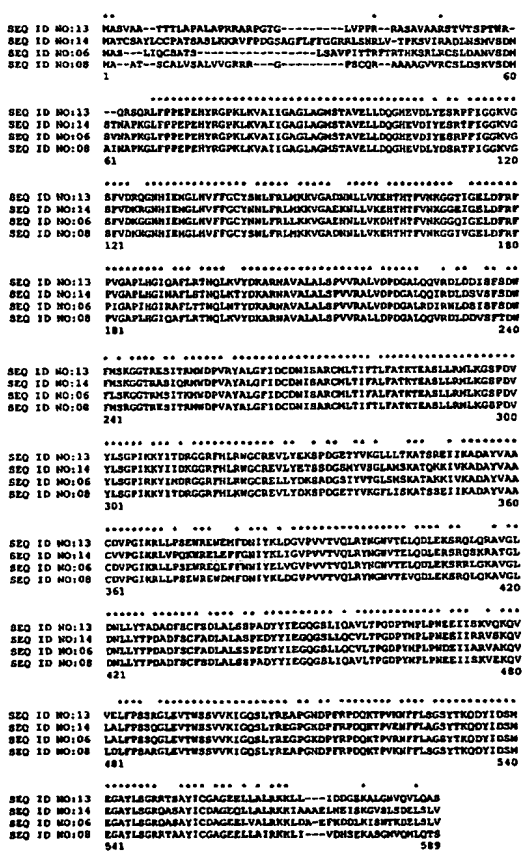
(54) Title: CAROTENOID BIOSYNTHESIS ENZYMES

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a carotenoid biosynthetic enzyme. The invention also relates to the construction of a chimeric gene encoding all or a portion of the carotenoid biosynthetic enzyme, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the carotenoid biosynthetic enzyme in a transformed host cell.



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/08746

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N9/02 C12N5/10 G01N33/50
//A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCOLNIK, P.A. AND BARTLEY, G.E.: "nucleotide sequence of zeta-carotene desaturase (Gen Bank U38550) from Arabidopsis thaliana" EMBL SEQUENCE DATA LIBRARY, 10 November 1995 (1995-11-10), XP002128769 heidelberg, germany accession no. U38550 ---	1-3,5
X	AL-BABILI, S., ET AL.: "a cDNA encoding for beta Carotene Desaturase (Accession no. AJ224683) from Narcissus pseudonarcissus" EMBL SEQUENCE DATA LIBRARY, 2 March 1998 (1998-03-02), XP002128770 heidelberg, germany accession no. AJ224683 --- -/--	1-3,5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 January 2000	18.02.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

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C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALBRECHT, M., ET AL.: "molecular cloning and functional expression in E. coli of a novel plant enzyme mediating zeta-carotene desaturation" FEBS LETTERS, vol. 372, 1995, pages 199-202, XP002112107 abstract, page 199; page 200, right column; Table 1; Fig. 1 ---	3
X	YAMAMOTO, K. AND SASAKI, T.: "rice cDNA from callus" EMBL SEQUENCE DATA LIBRARY, 6 August 1997 (1997-08-06), XP002128771 heidelberg, germany accession no. C27136 ---	1-3
X	US 5 539 093 A (FITZMAURICE WAYNE P ET AL) 23 July 1996 (1996-07-23) the whole document ---	6-13
X	LI, Z-H., ET AL.: "cloning and characteriaztion of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway" PLANT MOLECULAR BIOLOGY, vol. 30, 1996, pages 269-279, XP002112111 the whole document ---	6-11
X	AL-BABILI, S., ET AL.: "a novel, soluble form of phytoene desaturase from Narcissus pseudonarcissus chromoplasts is Hsp70-complexed and competent for flavinylation, membrane association and enzymatic activation" THE PLANT JOURNAL, vol. 9, no. 5, 1996, pages 601-612, XP002128744 the whole document	6-10
X	& AL-BABILI,S., ET AL.: "a novel, soluble form of phytoene desaturase from Narcissus pseudonarcissus chromoplasts is Hsp70-complexed and competent for flavinylation, membrane association and enzymatic activation" EMBL SEQUENCE DATA LIBRARY, 18 April 1995 (1995-04-18), heidelberg, germany accession no.X78815.1 --- -/--	6-10

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/08746

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALBRECHT, M., ET AL.: "biochemical characterization of purified zeta-carotene desaturase from Anabaena PCC7120 after expression in Escherichia coli" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 236, 1996, pages 115-120, XP002112340 the whole document	16
X	SANDMANN,G. , ET AL.: "a new non-radioactive assay of phytoene desaturase to evaluate bleaching herbicides" ZEITSCHRIFT FÜR NATURFORSCHUNG, vol. 51, July 1996 (1996-07), pages 534-538, XP002112339 the whole document	16
A	BARTLEY, G.E. AND SCOLNIK, P.A.: "Plant carotenoids: pigments for photoprotection, visual attraction , and human health" THE PLANT CELL, vol. 7, July 1995 (1995-07), pages 1027-1038, XP002112110 page 1027, right column; page 1030, right column; page 1035, Table 1	1-5, 11-16
A	BREITENBACH, J., ET AL.: "a higher-plant type zeta-carotene desaturase in the cyanobacterium Synechocystis PCC6803" PLANT MOLECULAR BIOLOGY, vol. 36, March 1998 (1998-03), pages 725-732, XP002112112 abstract, page 725, right column; page 727; Fig. 2 + 4	1-5, 11-16
A	BARTLEY, G.E., ET AL.: "molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 88, August 1991 (1991-08), pages 6532-6536, XP002128745 the whole document	1-5, 11-16
A	WO 95 34668 A (BIOSOURCE TECH INC) 21 December 1995 (1995-12-21) the whole document	1-16
A	WO 98 06862 A (SHEWMAKER CHRISTINE K ;CALGENE INC (US)) 19 February 1998 (1998-02-19) the whole document	1-16

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3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/08746

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SHOEMAKER, R., ET AL.: "public soybean EST project" EMBL SEQUENCE DATA LIBRARY, 17 March 1999 (1999-03-17), XP002128772 heidelberg, germany accession no. AI495658 ---	1-3,5
P,X	LUO, R. AND WURTZEL, ET.: "untitled" EMBL SEQUENCE DATA LIBRARY, 6 January 1999 (1999-01-06), XP002128773 heidelberg, germany cited in the application accession no. AF047490 ---	1-3,5
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3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US 99/08746

Box I Observations where c rtain claims w re f und uns archable (Continuation of it m 1 f first sh t)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/08746

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-5,11-16 partially

Isolation of partial rice-specific cDNAs encoding zeta-carotene desaturases; namely SEQIDs 1 and 3, furthermore the corresponding deduced amino acid sequences SEQIDs 2 and 4.

2. Claims: 1-5,11-16 partially

Isolation of a partial soybean-specific cDNA encoding zeta-carotene desaturase; namely SEQIDs 5, furthermore the corresponding deduced amino acid sequence SEQIDs 6.

3. Claims: 1-5,11-16 partially

Isolation of a full-length wheat-specific cDNA encoding zeta-carotene desaturase; namely SEQIDs 7, furthermore the corresponding deduced amino acid sequence SEQIDs 8.

4. Claims: 6-10,11-16 partially

Isolation of a full-length rice-specific cDNA encoding Phytoene desaturase; namely SEQIDs 9, furthermore the corresponding deduced amino acid sequence SEQIDs 10.

5. Claims: 6-10,11-16 partially

Isolation of a full-length wheat-specific cDNA encoding Phytoene desaturase; namely SEQIDs 11, furthermore the corresponding deduced amino acid sequence SEQIDs 12.

INTERNATIONAL SEARCH REPORT

rmation on patent family members

International Application No
PCT/US 99/08746

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